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Development and perspectives of fluorescent receptor assays

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Chapter 8

A novel fluorescent receptor assay for benzodiazepines, using coumarin-labeled desethylflumazenil as ligand

8.1 Introduction

Receptor assays can be used as an analytical tool to rapidly detect and quantitate drugs and related substances in biological matrices. The principle of the method is based on the competition of a labeled ligand and the analyte for binding to a certain receptor. Up till now, receptor assays are mostly performed with radioactive ligands. Radioactive ligands can be selectively measured at very low levels due to their high specific activity. Besides, since most of the radioisotopes are incorporated in the molecule, this type of labeling has no influence on the binding affinity of the ligand towards the receptor. However, since the use of radioactivity also has several disadvantages, non-radioactive ligands, such as fluorescent-labeled ligands, have been synthesized for different receptor types.

For the benzodiazepine receptor, these fluorescent-labeled ligands have been used for the characterization of the receptor itself [1-3] as well as for a quantitative benzodiazepine receptor assay [4-6]. However, the above ligands and the assays based thereupon have a number of drawbacks in terms of sensitivity and interference by matrix compounds or receptor proteins. Therefore we synthesized and evaluated a number of novel fluorescent ligands (Chapters 2 and 3). In Chapter 4, we concluded that coumarin-labeled desethylflumazenil, Mmc-O-CO-CH₂)₃-Ro15-3890 (CLDEF), is the fluorescent-labeled benzodiazepine of choice for use as labeled ligand in the fluorescent receptor assay for benzodiazepines.

In this Chapter, we describe a fluorescent receptor assay for benzodiazepines with CLDEF as the labeled ligand and in which the bound and unbound fraction of CLDEF are separated by filtration. Although solubilized benzodiazepine receptors showed lower background fluorescence (Chapter 5), it was still not possible to measure CLDEF in the presence of receptor material. So, we used membrane-bound receptors and dissociated CLDEF before quantitating the bound CLDEF. This was achieved by incubating the CLDEF bound to the receptors on the filter with a weak acetate buffer (pH 4) after the first filtration. The second filtrates then contained the previously bound CLDEF. For the quantitation of the bound fractions of CLDEF, we used a RP-HPLC system with a fluorescence detector. This was done because the filtrates also contained some impurities from the receptor material and also because fluorescence detectors for chromatographic purposes appeared to be more sensitive than conventional, static fluorescence detectors.

The binding of CLDEF towards the benzodiazepine receptor was established by performing saturation experiments. Further, calibration curves of three benzodiazepines (diazepam, lorazepam and flumazenil, respectively) were determined with CLDEF as label. The calibration curve of lorazepam was also compared with a calibration curve obtained with the radioactive-labeled ligand [³H]flunitrazepam.

8.2 Materials and Methods

Chemicals

[N-methyl- ^3H] flunitrazepam (82.0 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Lorazepam was a gift from Wyeth Laboratoria (Hoofddorp, The Netherlands). Flumazenil and diazepam were gifts from Roche Nederland (Mijdrecht, The Netherlands). The synthesis and purification of the fluorescent ligand (CLDEF) was described in Chapter 3 and its structure is shown in Figure 8.1. Methanol and acetonitrile were of hplc-grade and obtained from Lab-Scan (Dublin, Ireland). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

The MultiScreenAssay[®] Assay System and MultiScreen-FB filtration plates were kindly donated by Millipore (Etten-Leur, The Netherlands). Rialuma, used as scintillation cocktail, was obtained from Lumac (Olen, Belgium). Demineralized water was further purified by an Elgastat Maxima instrument (Elga, High Wycombe, UK) before use in the buffers.

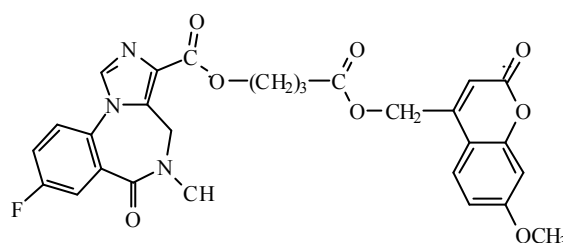


Figure 8.1 Structure of Mmc-O-CO-(CH₂)₃-Ro15-3890 (CLDEF).

HPLC-system

The chromatographic system consisted of a SP 8800 HPLC-pump (Spectra Physics, San Jose, CA, USA), an autosampler model 460 fitted with a 120 μl loop (Kontron Instruments, Basle, Switzerland), a F-1080 fluorescence detector, equipped with a 40 μl cuvette and 30 nm slit (Merck-Hitachi, Darmstadt, Germany) and a 2 bar backpressure regulator (Merck, Darmstadt, Germany). Peak heights were obtained from the chromatograms. Separation was performed using a 125 \times 4 mm i.d. column packed with 5 μm LiChrospher[®] 100 RP-18 (Merck, Darmstadt, Germany). The mobile phase consisted of 40% demineralized water, 40% methanol and 20 % acetonitrile. The flow rate was 1.0 ml/min. Mmc-CO-(CH₂)₃-Ro15-3890 (CLDEF) was detected at λ_{ex} 318 nm and λ_{em} 400 nm.

Preparation of membrane-bound receptors

Calf brains, obtained from the slaughterhouse and stored at -80°C after discarding the cerebella, were homogenized in 6 volumes (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i. Breisgau, Germany) fitted with a teflon pestle and centrifuged for 10 min at 1,000 \times g in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands). The supernatant was centrifuged for 60 min at 100,000 \times g. The resulting pellet (P₂) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged for 30 min at 100,000 \times g. This washing step was repeated four times. All operations were performed at 4°C . The washed P₂-pellet was resuspended in 5 volumes (w/v) phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkerød, Denmark). The lyophilized P₂-pellet was stored at -20°C . For the receptor binding assays, the lyophilized P₂-pellet was resuspended in Tris-HCl buffer (pH 7.4; 50 mM) with the glass-teflon Potter homogenizer (3.5 mg/ml). Protein amounts were determined according to the method described in earlier experiments [7] with bovine serum albumin as standard.

Radiolabeled binding assay

The filters of a MultiScreen-FB filtration plate were pre-wetted by pipetting 200 μ l ice-cold Tris-HCl buffer (pH 7.4; 50 mM) into each well. After waiting for at least 5 sec, vacuum was applied by the MultiScreen vacuum manifold (Millipore, Etten-Leur, The Netherlands). For the binding assay, 25 μ l Tris-HCl buffer, containing lorazepam (30 pM - 100 nM final concentration) and 25 μ l [3 H]flunitrazepam solution (2.2 nM final concentration) in Tris-HCl buffer were pipetted in duplicate into the wells of the filtration plate. To this mixture, 200 μ l receptor suspension were added and the plate was shaken for 1 min. After the incubation for 45 min at 4°C, vacuum was applied (400 mbar) and the filters were rinsed once with 200 μ l ice-cold buffer. To dissociate the bound [3 H]flunitrazepam, 200 μ l acetate buffer (pH 4; 100 mM) were pipetted in each well and incubated for 20 min at room temperature. Thereafter, the dissociation solutions were collected in a microtiterplate by filtration (vacuum, 400 mbar). Hundred μ l of the filtrates were transferred into 6 ml polyethylene counting vials and dispersed in 3.5 ml Rialuma. After shaking the vials, the radioactivity was counted for 5 min in a Tri-Carb 4000 Packard scintillation counter (Canberra Packard, Groningen, The Netherlands). The binding experiments were performed in duplicate.

Fluorescent-labeled binding assay

For the saturation experiments, 25 μ l CLDEF solution (0.5 - 50 nM final concentration) in Tris-HCl buffer (pH 7.4; 50 mM) were pipetted in duplicate in the wells of the filtration plate after pre-wetting the filters with 200 μ l ice-cold Tris-HCl buffer and filtrating the latter. For the determination of total binding, 25 μ l Tris-HCl buffer, and for the determination of non-specific binding, 25 μ l Tris-HCl buffer containing 100 μ M flumazenil, were added, respectively. To this mixture, 200 μ l of the receptor suspension (225 μ g protein per assay) were added and the plate was shaken for 1 min. After incubation for 45 min at 4°C, vacuum was applied (400 mbar) and the filters were rinsed once with 200 μ l ice-cold buffer. To dissociate the bound CLDEF, 200 μ l acetate buffer (pH 4; 100 mM) were pipetted in each well and incubated for 20 min at room temperature. Thereafter, the dissociation solutions were collected in a microtiterplate by filtration (vacuum, 400 mbar) and transferred in 300 μ l glass microvials (Phase Sep, Waddinxveen, The Netherlands). Hundred μ l of the filtrates were analyzed with the above described HPLC-method.

To quantitate the amount of CLDEF in the filtrates, five different standard solutions were made by diluting CLDEF in Tris-HCl buffer with acetate buffer to get the final concentrations of 0.3; 0.5; 1.5; 3.0 and 5.0 nM CLDEF, respectively. These samples were analyzed in duplicate.

For the calibration curves, for which the procedure was similar to that used for the saturation experiments, 25 μ l CLDEF solution (7.3 nM final concentration) in Tris-HCl buffer was mixed with 25 μ l of a solution of diazepam (1 nM - 1 μ M final concentration), of lorazepam (0.3 nM - 300 nM final concentration), or of flumazenil (30 pM - 30 nM final concentration).

8.3 Results and discussion

Principle of the fluorescent receptor assay

The principle of the fluorescent receptor assay using the microtiter filtration plate is shown in Figure 8.2. After separation of the bound and unbound fractions of CLDEF by filtration in step 3, the bound CLDEF is dissociated from the benzodiazepine receptor in steps 5 and 6. This is done to obtain the CLDEF in a solution, free from receptors and filter materials, so that the amount of CLDEF can be determined by measuring the fluorescent signal without interference or quenching.

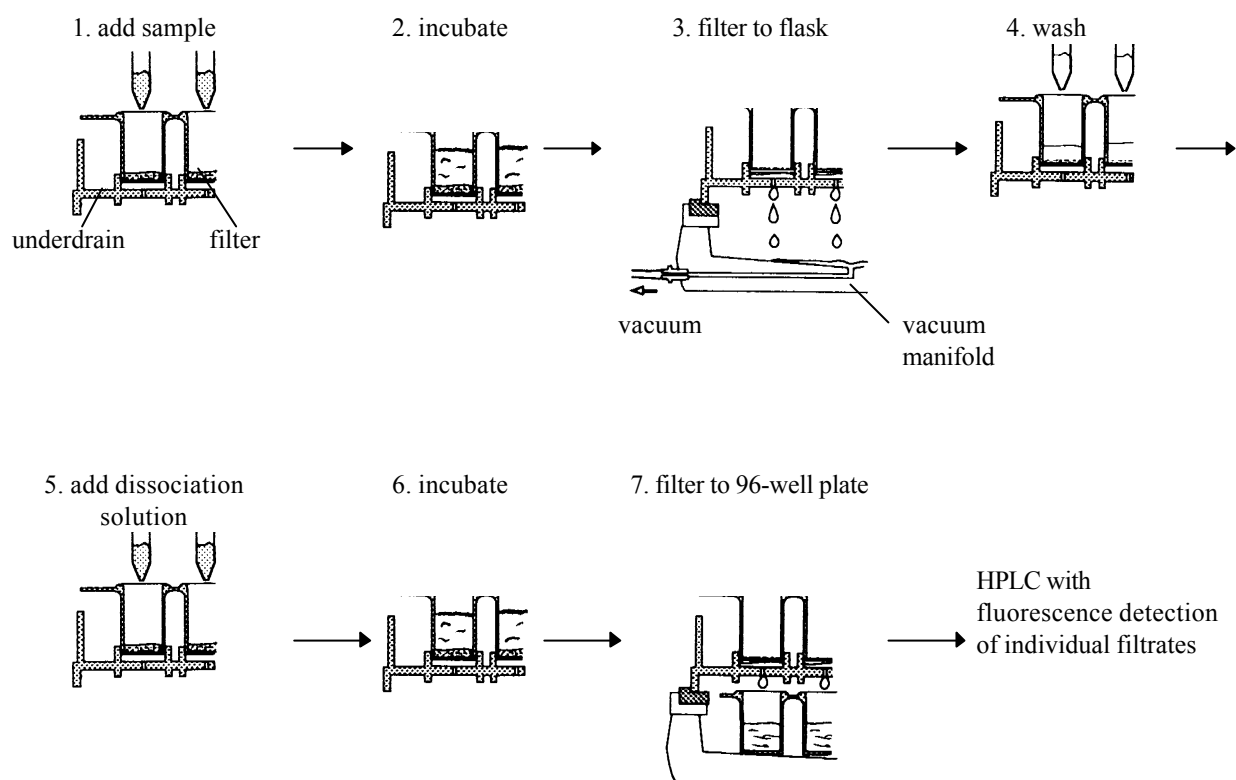


Figure 8.2 Principle of the fluorescent receptor assay for benzodiazepines, using the MultiScreen Assay System.

Instead of measuring the bound fraction of CLDEF, it is also possible to determine the free fraction of CLDEF. In this case, the filtrate has to be collected during filtration step 3. Since only 26% of the added CLDEF at an assay concentration of 7.3 nM is usually bound to the receptors (total binding), we preferred to quantitate the bound fractions of CLDEF in order to enable more precise quantitation of the analytes [8].

In the fluorescent benzodiazepine receptor assays developed by Takeuchi et al. [4,5], the free label fractions were quantitated after collecting these fractions by centrifugation. McCabe et al. [2] and Havunjan et al. [1] quantitated the bound fractions of their labeled ligands. However, they performed their measurements in the presence of the receptor materials. This is rather surprising, since we observed considerable background fluorescence from receptor materials. We decided to measure the bound fractions of CLDEF in the absence of receptor material, to obtain higher sensitivity and precision.

The HPLC-system

We quantitated the amount of bound CLDEF after hydrolysis by HPLC. This was done because the fluorescence detectors available for chromatographic purposes were found to be more sensitive than static fluorescence spectrometers using cuvettes. Besides, after the dissociation, the dissociation solutions contained fluorescent interferences. For this reason, during the preparation of the membrane-bound receptor material, the P₂-pellet was washed 5 times instead of the 2 times according to our standard procedure [7]. By using an HPLC-system, CLDEF can be separated from the remaining interferences.

The mobile phase composition of the chromatographic system was obtained from the optimization experiments for the purification of CLDEF [9]. With this system, CLDEF has a capacity factor of 4.5 and is completely resolved from the interferences.

In previous experiments, we examined the dissociation of the bound CLDEF from the benzodiazepine receptors [10]. The highest recovery ($90.8\% \pm 0.39$ of CLDEF) was achieved by dissociation with Tris-HCl buffer (pH 7.4; 50 mM), containing 10 μ M flumazenil. To quantitate the dissociated CLDEF, 100 μ l of the dissociation solution was injected directly into the HPLC-system. However, Tris-HCl buffer gave an enormous solvent peak, so that there was insufficient resolution between the solvent peak and CLDEF.

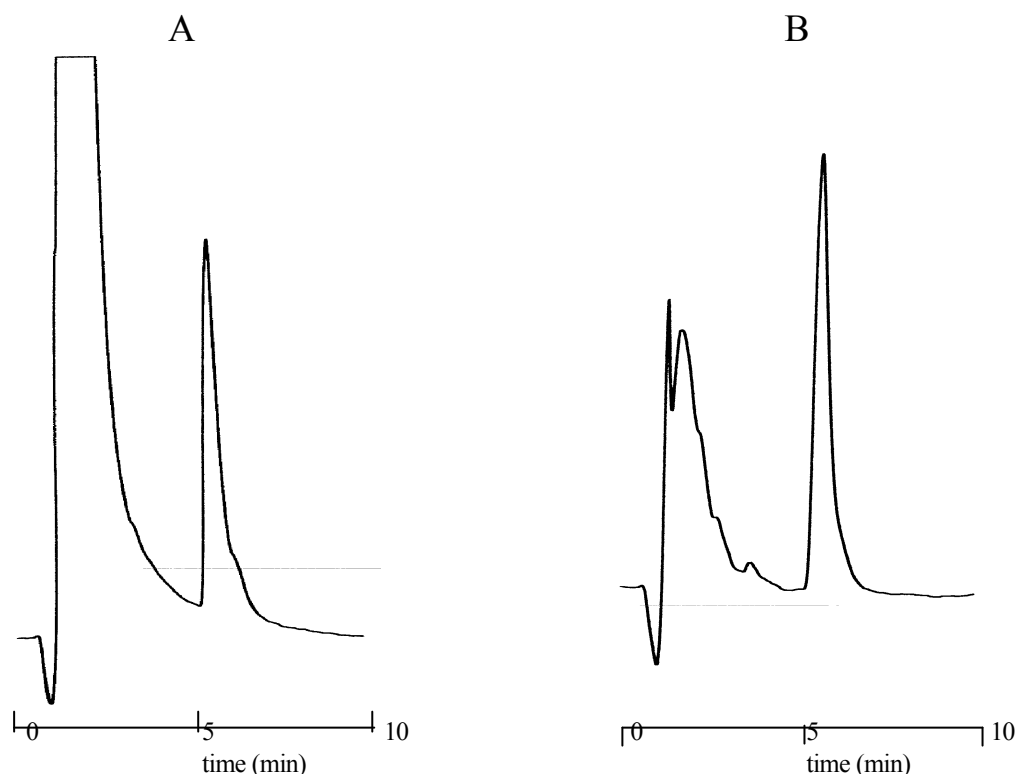


Figure 8.3 Chromatograms of 10 nM CLDEF, (A) in Tris-HCl buffer (pH 7.4; 50 mM), containing 10 μ M flumazenil and (B) in acetate buffer (pH 4; 100 mM).

Acetate buffer (pH 4; 100 mM) was found to be a suitable alternative to dissociate the bound CLDEF from the receptors, with a recovery of $87.2\% \pm 1.16$ and the resulting solvent peak did not interfere with the measurement of the dissociated CLDEF. Figure 8.3 shows chromatograms of 10 nM solutions of CLDEF in Tris-HCl buffer and acetate buffer, respectively.

Binding characteristics of CLDEF to the benzodiazepine receptor

The saturation and inhibition curves for the radioactive labeled benzodiazepine [3 H]flunitrazepam and the fluorescent labeled benzodiazepine, CLDEF, were fitted with the program EBDA-Ligand V4 (Biosoft, Cambridge, UK) [11], using a one-binding site model. Figure 8.4 shows a representative saturation curve of CLDEF. The specific binding was calculated by subtracting the non-specific binding from the total binding. The binding affinity, K_i , of CLDEF for the benzodiazepine receptor was calculated to be 8.60 ± 2.89 nM. This value is comparable to the affinity constant estimated with radioligand binding assays. Inhibition assays with [3 H]flunitrazepam gave a K_i -value of 6.5 nM for CLDEF [9]. The B_{\max} value for CLDEF was calculated to be 3.42 ± 0.01 nM, which corresponds to 3.84 ± 0.09 pM/mg protein. In a previous study, we established a B_{\max} of 1.0 pM/mg protein using the radioactive ligand [3 H]flunitrazepam [7]. We do not have an explanation for this difference in B_{\max} -values. The increase in binding sites observed in the present study cannot be assigned to extra binding sites for the fluorophore, or to flumazenil having more binding sites than flunitrazepam, since CLDEF could be displaced completely by diazepam, lorazepam and flumazenil (see Figure 8.5).

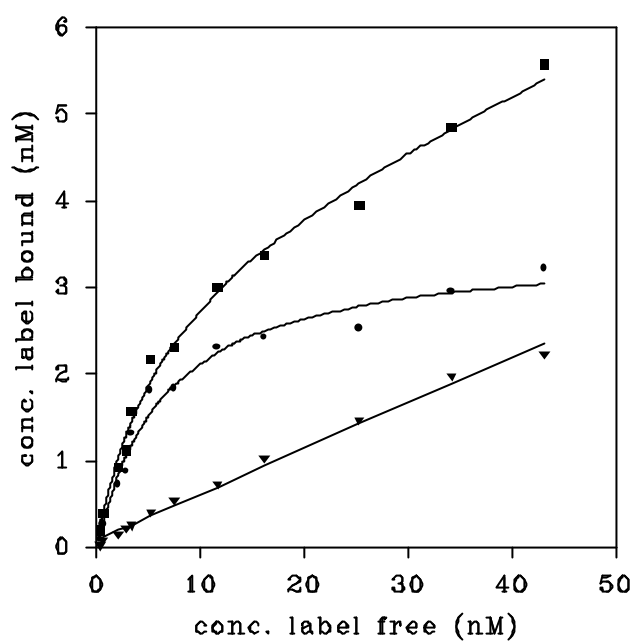
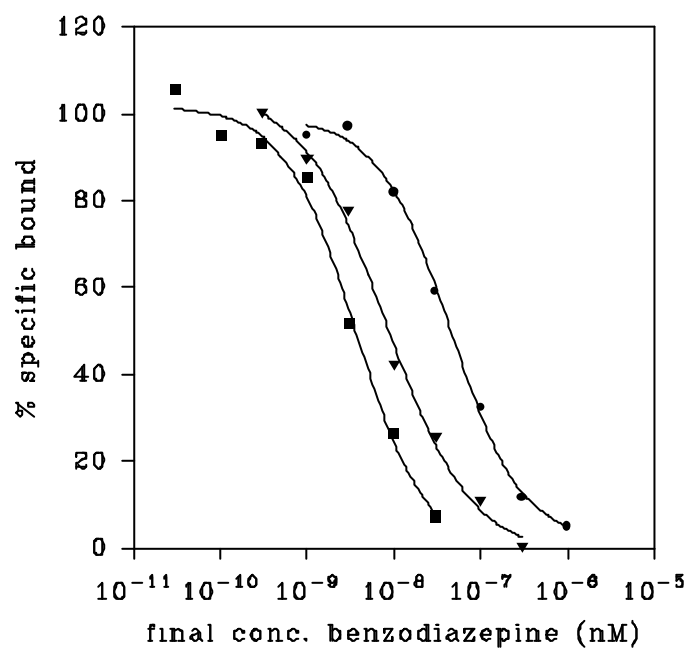


Figure 8.4 Saturation curve of CLDEF for the benzodiazepine receptor. \blacksquare represents the total binding, \bullet the specific binding and \blacktriangledown the non-specific binding, respectively.

Figure 8.5 Calibration curves of different benzodiazepines using CLDEF as the fluorescent ligand: diazepam



(Δ), lorazepam (◊) and flumazenil (▽).

Havunjian et al. [1] also found a higher B_{\max} for the fluorescent-labeled flumazenil derivative (NBD-NH-(CH₂)₃-Ro15-3890 (BD 623), than for [³H]flumazenil, namely 7.2 versus 2.3 nM. As a possible explanation, they mentioned the difference in separation of bound and unbound fractions of labeled ligand between their two methods: Using [³H]flumazenil, they used filtration, but with BD 623, they did not separate the bound and unbound fractions. After binding of BD 623 towards the receptor, the fluorescence intensity decreased and from the degree of fluorescence quenching the amount of bound BD 623 was estimated. Yet, this hypothesis was not investigated further.

The non-specific binding of CLDEF was 25% of the total binding at a free initial concentration of 15 nM and was comparable with other fluorescent receptor assays for benzodiazepines. Takeuchi et al. [7] found a non-specific binding of 27% in their time-resolved fluorometric assay with Eu-1012-S as labeled-benzodiazepine and a non-specific binding of 20% in their fluorescent receptor assay with Ro7-1986-AMCA as labeled ligand [4].

Table 8.1 IC₅₀ values of various benzodiazepines, determined using the fluorescent receptor assay (n=2).

	IC ₅₀ (nM) (± SD)
flumazenil	4.9 (± 1.5)
lorazepam	7.2 (± 0.5)
diazepam	41 (± 7)

Fluorescent receptor assay for benzodiazepines

CLDEF was tested as fluorescent label by determining calibration curves for three benzodiazepines with different affinities for the benzodiazepine receptor, namely flumazenil, lorazepam and diazepam. These experiments were done with a CLDEF assay concentration of 7.3 nM. This concentration corresponds with the K_d of CLDEF. Representative calibration curves are shown in Figure 8.5.

With CLDEF, the three benzodiazepines showed the same order in affinity as when determined with a radioactive labeled ligand. The IC₅₀-values, calculated from the calibration curves, are presented in Table 8.1.

To compare the FRA with RRA, calibration curves of lorazepam were determined with the fluorescent label CLDEF as well as with the radioactive label [³H]flunitrazepam.

In order to compare the IC₅₀-values of lorazepam, determined with two different ligands, the ratio between the concentration of free labeled ligand (L^*) and K_d of the labeled ligand has to be equal in the two assays, since the relation between the IC₅₀ and the K_i is expressed by the Cheng-Prusoff equation [12]:

$$IC_{50} = K_i * (1 + \frac{[L^*]}{K_d}) \quad (8.1)$$

In our experiments, this requirement was fulfilled for both ligands.

As can be seen in Figure 8.6, the two curves are very similar. The IC₅₀ of lorazepam determined with [³H]flunitrazepam, was 6.6 ± 0.7 nM and did not differ significantly from the IC₅₀, determined in the fluorescent receptor assay (7.2 ± 0.5 nM) when compared with Student's t-test ($p=0.45$). This proves that receptor assays can be performed successfully with fluorescent labels.

In order to obtain an impression of the accuracy and precision of the FRA, three lorazepam samples used for the fitting of the calibration curves were back-calculated to their original concentrations. Accuracy and precision data were determined (Table 8.2) and compared with those from the RRA. As can be seen, the data of both receptor assays are identical.

Takeuchi et al. [5] developed a time-resolved fluorometric assay for benzodiazepines. They separated the bound and free fractions of their label, Eu-1021-S, by centrifugation and quantitated the free fractions by measuring the time-resolved fluorescence in the supernatant.

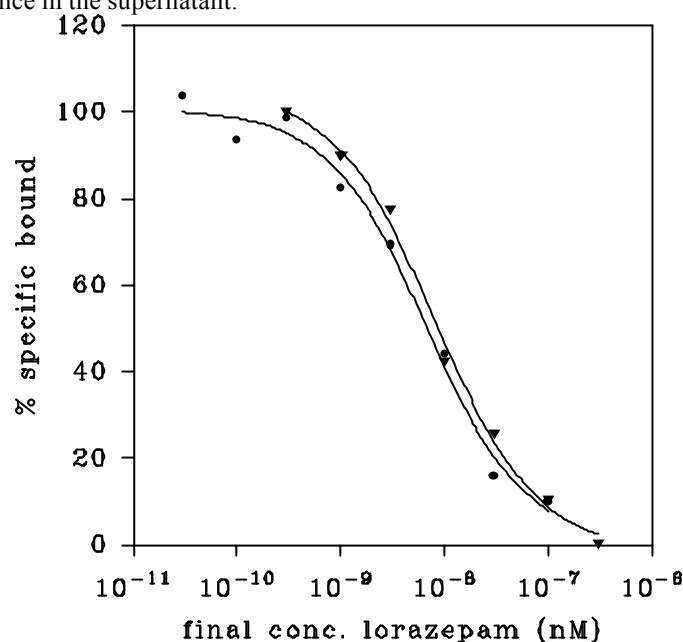


Figure 8.6 Calibration curves of lorazepam with different types of labeled ligands: ● represents an assay performed with [^3H]flunitrazepam (2.2 nM final concentration) and ▲ represents an assay performed with CLDEF (7.3 nM final concentration).

They chose a europium chelate as a label, since their supernatant exhibited strong background fluorescence which interfered with the measurement of the fluorophore-labeled ligands. By using an HPLC-system for the quantification of CLDEF, we circumvented the occurrence of background fluorescence. Besides, in our assay it was possible to determine the bound fractions instead of the unbound fractions, which improved the precision of the assay. Takeuchi and Rechnitz [4] also used an HPLC-system in their FRA, with AMCA-Ro7-1986 as ligand, but they quantitated the free fraction of the ligand. To obtain sufficient difference in the fluorescence signal between the maximal binding and the non-specific binding, they used a high amount of receptor material, namely 40 mg/ml. Ensing [13] calculated that increasing the receptor concentration will have a negative effect on the detection limit.

Table 8.2 Accuracy (in %) and precision of the back-calculated concentrations of three lorazepam concentrations in Tris-HCl buffer (50 mM, pH 7.4) (n=2) assayed by FRA and RRA.

conc. in buffer (nM)	FRA (\pm SD)	RRA (\pm SD)
30	91.3 (\pm 3.9)	103.2 (\pm 7.2)
100	113.3 (\pm 10.2)	90.9 (\pm 6.1)
300	85.5 (\pm 2.6)	115.8 (\pm 24.4)

McCabe et al. [2] also measured the bound fractions of their fluorescent-labeled benzodiazepine, fluorescein-NH-(CH₂)₃-Ro15-3890 (BD 621). After separation of the bound and unbound fractions by centrifugation, they resuspended the pellet in buffer and measured the fluorescence intensity of the suspension during which BD 621 was still bound to the receptor. The fluorescein fluorophore has higher excitation and emission wavelengths ($\lambda_{\text{ex}} = 499 \text{ nm}$ and $\lambda_{\text{em}} = 521 \text{ nm}$) than our fluorophore, a coumarin derivative. Although the autofluorescence of the receptor material and for the background is less at higher wavelengths, the interferences may still be substantial and will also have a detrimental effect on the sensitivity of the assay. Havunjian et al. [1] measured the bound fraction of their fluorescent-labeled benzodiazepine, NBD-NH-(CH₂)₃-Ro15-3890 (BD 623). After binding of NBD-NH-(CH₂)₃-Ro15-3890 to the benzodiazepine receptor, the fluorescence intensity was decreased. In this assay, the fraction bound was quantitated from the amount of quenching observed. The advantage of this method is that there is no need for separation of the bound and unbound fractions of NBD-NH-(CH₂)₃-Ro15-3890. Yet, it will be clear that the sensitivity and precision of this method will diminish when low levels of benzodiazepines are to be assayed, also if one considers the low relative fluorescence of the NBD fluorophore, which is only 30% of that of CLDEF.

8.4 Conclusions

The benzodiazepine receptor assay can be performed successfully with the fluorescent labeled benzodiazepine CLDEF. The IC₅₀-values of lorazepam, which are an indication for the sensitivity of the receptor assays, were virtually identical for the FRA and the RRA, namely $7.2 \pm 0.5 \text{ nM}$ and $6.6 \pm 0.7 \text{ nM}$, respectively. Also, the validation of the lorazepam calibration curve of both methods showed comparable accuracy and precision data. In our FRA, the bound fractions of the labeled ligand were quantitated instead of the unbound fractions, which is favourable for the precision of the assay. To avoid the interference of background signals caused by autofluorescence of the receptor material, the bound fractions of the CLDEF were dissociated from the receptors after the filtration. The second filtrates, containing the bound fractions in absence of receptor material, were analyzed by an HPLC-system. The use of this HPLC-system was essential since even the second filtrates gave a relative high fluorescence background signal. Yet, the use of an HPLC-system for the quantitation has no negative influence on the time necessary for the receptor assay: The time to perform a FRA and to quantitate the bound fractions is comparable with the time necessary for a RRA. Also, the sensitivity of the flow-through fluorescence detector coupled to the HPLC system was found to be better than of various static fluorescence detectors.

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References

- [1] R.H. Havunjian, B.R. de Costa, K.C. Rice and P. Skolnick. Characterization of benzodiazepine receptors with a fluorescence-quenching ligand, *J. Biol. Chem.*, 265 (1990) 22181-22186.
- [2] R.T. McCabe, B.R. de Costa, R.I. Miller, R.H. Havunjian, K.C. Rice and P. Skolnick. Characterization of benzodiazepine receptors with fluorescent ligands, *FASEB J.*, 4 (1990) 2934-2940.
- [3] J.L. Velazquez, C.L. Thompson, E.M. Barnes and K.J. Angelides. Distribution and lateral mobility of GABA/benzodiazepine receptors on nerve cells, *J. Neurosci.*, 9 (1989) 2163-2169.
- [4] T. Takeuchi and G.A. Rechnitz. Nonisotopic receptor-binding assay for benzodiazepine receptors utilizing a fluorophore labeled ligand, *Anal. Biochem.*, 194 (1991) 250-255.
- [5] Takeuchi, T. Nishikawa, R. Matsukawa and J. Matsui. Nonisotopic receptor assay for benzodiazepine drugs using time-resolved fluorometry, *Anal. Chem.*, 67 (1995) 2655-2658.
- [6] T. Takeuchi, M. Yoshida, Y. Kabasawa, R. Matsukawa, E. Tamiya and I. Karube. Time-resolved fluorescence receptor assay for benzodiazepines, *Anal. Lett.*, 26 (1993) 1535-1545.
- [7] M.J. Janssen, M. Stegeman, K. Ensing and R.A. de Zeeuw. Solubilized benzodiazepine receptors for use in receptor assays, *J. Pharm. Biomed. Anal.*, 14 (1996) 989-996.
- [8] J. Šmisterová, K. Ensing and R.A. de Zeeuw. Methodological aspects of quantitative receptor assays, *J. Pharm. Biomed. Anal.*, 12 (1994) 723-745.
- [9] Chapter 3, this thesis.
- [10] Chapter 7, this thesis.
- [11] P.J. Munson and D. Rodbard. Ligand: A versatile computerized approach for characterization of ligand-binding systems, *Anal. Biochem.*, 107 (1980) 220-239.
- [12] Y.-C. Cheng and W.H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction, *Biochem. Pharmacol.*, 22 (1973) 3099-3108.
- [13] K. Ensing. The radioreceptor assay, a tool for the bioanalysis of drugs, in: *Bioanalysis of anticholinergics with muscarinic receptors in relation with chronic obstructive lung diseases*, Ph.D. thesis, University of Groningen, 1984, pp 25-40.